The Total Reactive Antioxidant Potential (TRAP) and Total Antioxidant Reactivity (TAR) of *Ilex paraguayensis* Extracts and Red Wine

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A concentração de anti oxidantes em extratos de *Ilex paraguayensis* e de vinhos tintos foi determinada usando procedimentos baseados no amortecimento da quimiluminescência do luminol, induzida por radicais livres, e pela descoloração dos cátions radicais pré-formados derivados do ABTS. As metodologias deram resultados similares, permitindo a avaliação dos índices de potencial antioxidante reativo total (TRAP) e de reatividade antioxidante total (TAR). Os resultados obtidos empregando saquinhos de chá e folhas secas de diferentes marcas indicaram que as infusões de *Ilex paraguayensis* apresentam valores TRAP mais altos (em equivalentes Trolox) de 6.6 ± 1.3 mM (método ABTS) e 3.4 ± 1.1 mM (método Luminol). O TAR das amostras foi de 4.7 ± 1.5 mM. Estes resultados, assim como aqueles obtidos com o vinho tinto, indicam que ambos apresentam alta concentração de antioxidantes, com uma fração deles sendo constituída por compostos de alta reatividade frente a radicais livres.

The charge of antioxidants in *Ilex paraguayensis* extracts and in red wines were determined employing procedures based on the quenching of luminol chemiluminescence induced by free radicals and the bleaching of preformed radical cations derived from ABTS. Both methodologies give similar results, allowing the evaluation of the total reactive antioxidant potential (TRAP) and the total antioxidant reactivity (TAR) indexes. The results obtained employing both tea bags and dry tea leaves from different trademarks indicate that *Ilex paraguayensis* infusions present large TRAP values (in Trolox equivalents) of 6.6 ± 1.3 mM (ABTS method) and 3.4 ± 1.1 mM (luminol method). The TAR of the samples was 4.7 ± 1.5 mM. These results, as well as those obtained for red wine, indicate that both beverages present high antioxidant concentrations, a fraction of which is constituted by compounds of high reactivity towards free radicals.

Keywords: total antioxidant reactivity (TAR), total reactive antioxidant potential (TRAP), *Ilex paraguayensis*, antioxidant potential, red wine, antioxidant potential

Introduction

Adequate antioxidant levels are a pre-requisite for the normal functioning of aerobic cells and tissues. Decreased levels of antioxidants can be the origin or consequence of several pathologies, such that the evaluation of antioxidants provides useful index of the capacity of the organism to sustain conditions of oxidative stress. The importance of the defenses against deleterious free radical mediated processes has led to great efforts aimed towards the development of new antioxidant compounds and the evaluation of the antioxidant activity of natural compounds present in plant extracts. There exists great interest in developing

methods aimed at evaluating the total amount of antioxidants present in biological fluids¹⁻⁷ and in vegetal extracts⁸⁻¹¹. In general, these experimental procedures evaluate either the total amount of antioxidants or the capacity of the mixture to decrease the steady state free radical concentration. Most of the methods applied to biological fluids determine the total reactive antioxidant potential (TRAP) and correspond to the first category. We have recently proposed two methods that allow, under certain conditions, the simultaneous determination of TRAP values and the estimation of the reactivity of the antioxidants present in the samples. These experimental procedures are based on the quenching of the luminol luminescence induced by the

radicals produced in the thermal decomposition of an azo-compound¹², and on the bleaching of the absorption due to the presence of preformed radical cations derived from 2,2'-azinobis(3-ethylbenz-thiazolinesulfonic acid) (ABTS)¹³. In particular, the former procedure allows the definition of another index, the total antioxidant reactivity (TAR) of the sample, related to the capacity of the sample to decrease the steady state free radical concentration¹². In the present work, we report a comparison of the results obtained employing both methods regarding the total charge of antioxidants and their reactivity in complex mixtures. The systems selected for this study were red wine samples and infusions of *Ilex paraguayensis* St. Hil. var. *paraguayensis*.

Ilex paraguayensis St. Hil. var. *paraguayensis* is widely consumed as "yerba mate" in South America. "Yerba mate" is rich in hydrosoluble polyphenols, such as isochlorogenic acid, caffeic acid and chlorogenic acid¹⁴, which present high antioxidant capacities and can confer to their infusions a hepatoprotective role and reinforce the antioxidant defenses of the organism¹⁵. Similarly, it has been proposed that the antioxidants present in wine, especially in red wine, can contribute to the reported beneficial effects of ingesting this beverage upon the oxidation of low density lipoproteins and their pathological consequences¹⁶⁻²⁰. The total charge of antioxidants in wine has been recently reported²¹⁻²⁴, but there has not been an evaluation of their reactivity nor a comparison with the levels of antioxidants present in other beverages.

Experimental

Chemicals

ABTS diammonium salt from Boehringer Mannheim GmbH, 2,2'-azo-bis(2-amidinopropane) (ABAP) from Wako Chemical Co., luminol from Sigma, and Trolox from Aldrich Chemical Co. were employed as received. Chilean red wines of several types and trademarks were employed within two days of opening the bottle. Yerba mate infusions were prepared employing tea bags from four different Argentinian trademarks and loose leaves from two Argentinian and two Brazilian trademarks.

Methods

"Yerba mate" infusions were prepared by pouring 160 mL of hot water over a tea bag or ground commercial leaves. The extraction was carried out for a few seconds while gently shaking to obtain a solution with a concentration similar to that of a typical drinking infusion. The concentration of the solution was standardized by measuring the absorbance of the infusion (diluted 100 times) at the wavelength of maximum absorption (323 ± 2 nm).

Evaluation of the antioxidant capacity from the bleaching of ABTS derived radical cations

A solution of ABAP (2 mM) and ABTS (75 μ M) was incubated at 45 °C for the time necessary to reach an absorbance of ca. 0.35 at 734 nm, due to the production of the ABTS-derived radical cation. The solution was brought to 25 °C and a small (a few microliters) aliquot of the yerba mate infusion or red wine was added. The kinetics of the consumption of the radical cations was followed by the decrease in the absorbance at 734 nm. The data collection was started 7 s after the incorporation of the additives, and was carried out in an Shimadzu spectro-photometer UV-160. Trolox was employed as the reference inhibitor^{9,13}. All solutions and infusions were used freshly prepared or kept for less than 3 h at 0 °C until use. Phosphate buffer, 50 mM, pH 7.0, was employed as the solvent.

Evaluation of the antioxidant capacity by quenching luminol luminescence.

This experimental procedure was similar to that previously reported²⁵. The reaction mixture contained the free radical source (ABAP, 10 mM or 2 mM) and luminol (50 μ M) in buffer glycine (0.1 M), pH 8.6. The incubation of this mixture at 37 °C generates an almost constant light intensity that was directly measured in a Bio-Orbit 1250 luminometer with a thermostated cell holder. The content and reactivity of the antioxidants present in the yerba mate infusion or in the red wine was determined by evaluating the time course of the luminol luminescence after the addition of a small aliquot of the sample. Trolox was employed as the reference inhibitor¹².

Results and Discussion

Bleaching of preformed ABTS radical cations

The addition of free radical scavengers to a solution containing ABTS-derived radical cations leads to a decrease in the absorbance of the sample at 734 nm. Typical results obtained employing Trolox or a yerba mate infusion are given in Fig. 1. The addition of Trolox elicits an instantaneous decrease in absorbance that is linearly related to the Trolox concentration. This decrease can be associated with the consumption of ABTS radical cations in the process

that is complete in less than 7 s.

The absorbance decay following the yerba mate infusion is qualitatively different from that obtained employing Trolox (Fig. 1). The difference has been ascribed to the presence of efficient and inefficient antioxidants in the additive^{9,13,23}. The concentration of efficient antioxidants can be derived, in Trolox equivalents, from the extent of

Figure 1. Time course of the absorbance at 734 nm due to the ABTS derived radical cations following the addition of 5 μ L Trolox (●) or 3 μ L/mL of a yerba mate (V) extract (○).

the fast decay, while the change in the absorbance measured after a long time (arbitrarily chosen as 17 min) can be related to the total amount of antioxidants (efficient and inefficient) present in the sample. The decrease in absorbance elicited by the aliquot of the infusion ($(\Delta A)_{\text{infusion}}$) is

related to the equivalent Trolox concentration present in the infusion (TRAP) through

$$(\text{TRAP})_{\text{ABTS}} (\mu\text{M}) = \frac{[(\Delta A)_{\text{infusion}} / (\Delta A)_{\text{Trolox}}]}{\alpha} \quad (2)$$

where $(\Delta A)_{\text{Trolox}}$ is the decrease in absorbance elicited by the addition of 1 μ M of Trolox, and is a dilution factor defined by

$$\alpha = \frac{\text{Volume of the aliquot}}{\text{Volume of the sample}} \quad (3)$$

The values of the total TRAP evaluated by this procedure are collected in Table 1. This table also gives the value of the fraction of the antioxidants associated with the fast decay (f_{fast}). This value can be considered a measure of the distribution of the reactivities of the antioxidants present in the infusion. The values of the TRAP and f_{fast} previously reported for several Chilean red wines²³ have also been included in this table. The data presented in this table indicate that both yerba mate infusions and red wine present a substantial antioxidant capacity. For yerba mate, the values obtained for the infusions obtained from tea bags or

Table 1. Total TRAP (mM) and f_{fast} values derived from the bleaching of ABTS radical cation absorbance.

Beverage	Type	TRAP	TRAP/A ₃₂₃	f_{fast}
Yerba mate	tea bag (I)	6.8	14	0.51
	leaves (I)	6.6	20	0.40
	tea bag (II)	7.0	17	0.50
	leaves (II)	5.9	22	0.38
	tea bag (III)	7.7	15	0.50
	tea bag (IV)	8.4	17	0.45
	leaves (V)	3.8	20	0.46
	leaves (VI)	6.1	17	0.48
		6.6 $\pm$ 1.3	17 $\pm$ 3	0.47 $\pm$ 0.04
Red wine	Sauvignon (VII)	29.6		0.53
	Sauvignon (VIII)	29.0		0.55
	Sauvignon (IX)	28.2		0.55
	Sauvignon (X)	31.6		0.53
	Sauvignon (XI)	33.0		0.55
	Pinot (VII)	29.9		0.56
	Merlot (VII)	25.1		0.55
	Unspecified	26.8		0.54
		29.1 $\pm$ 2.3		0.54 $\pm$ 0.01
White wine	Sauvignon (VIII)	4.8		0.46

A₃₂₃ correspond to 10^{-2} times the absorbance measured at this wavelength.

The numbers between parentheses indicate different trademarks.

The error indicated corresponds to the standard deviation of the sample.

Data for red and white wines was taken from Ref. 23.

Figure 2. Time course of the luminol luminescence in the luminol/ABAP (2mM) system following the addition of a yerba mate (III) tea bag extract: (A) 1 μ L; (B) 5 μ L; (C) 10 μ L. The original extract ($A_{323}/100 = 0.5$) was diluted 10^3 times prior to the aliquot addition. The total volume of the sample was 1 mL.

The plot on the left shows the dependence of (I_0/I), where I_0 and I are the luminescence intensities prior to and after the aliquot addition, as a function of the aliquot size.

dry leaves are similar, and they are not very dependent upon the trademark employed. Furthermore, these values are considerably higher than those previously reported for black tea infusions (3.2 mM) and other infusions widely used in Chile⁹, including boldo (*Peumus boldus* Mol) infusions (2.1 mM), making yerba mate extracts very promising for performing *in vivo* studies of the capacity to diminish the effects of oxidative stress conditions.

The luminescence of luminol elicited by the radicals produced in the pyrolysis of ABAP can be quenched by free radical scavengers, allowing the definition of TRAP and TAR indexes for complex mixtures¹². The TAR index is obtained from the instantaneous decrease in luminescence associated with the incorporation of the additive, while the TRAP index is obtained from the induction time observed when higher concentrations of the antioxidants are incorporated into the ABAP/luminol system^{12,25}. The time profiles of the luminescence following the addition of yerba mate infusions or red wine are shown in Figs. 2 to 5. The data obtained employing yerba mate allow the evaluation of the TAR (Fig. 2) and TRAP (Fig. 3) indexes. The fact that the rates of the luminescence increase after the induction time is lowered can be explained in terms of the presence of rather high quantities of low efficiency antioxidants. The TRAP and TAR values obtained from these procedures are given in Table 2. Due to the ill-defined onset

of the luminescence increase, the induction time was taken as the time required to reach a luminescence intensity equal to 20% of that present prior to the incorporation of the additive.

The results obtained employing red wine are shown in Figs. 4 and 5. In particular, they allow a precise evaluation of the decrease in luminescence following the incorporation of the additive (Fig. 4). This is due to the slow recovery of the luminescence, and permits the evaluation of the TAR index. On the other hand, when larger aliquots are employed in order to evaluate the induction time, the recovery is so slow that it does not allow the definition of an induction time, as such precluding the evaluation of the TRAP by this procedure. This indicates that in red wine there exist very large quantities of low efficiency antioxidants which do not define an induction time under the conditions employed²⁷.

The results given in Table 2 are qualitatively similar to those obtained by measuring the bleaching of the pre-formed ABTS radical cation. The results in this Table show that all the yerba mate extracts present a similar TRAP capacity (3.4 ± 1.1 mM), which is nearly two factors smaller than that measured by the ABTS method (6.6 ± 1.3 mM). Also, the data in this Table show a fairly good correlation between the TAR and TRAP values of the different infusions. With regard to the antioxidant capacity

Figure 3. Time course of the luminol luminescence in the luminol/ABAP (10 mM) system following the addition of the yerba mate (III) tea bag extract: (A) 5 μL ; (B) 7.5 μL ; (C) 10 μL . The original extract was diluted 10^2 times prior to the aliquot addition. The total volume of the sample was 1 mL. The plot on the left shows the dependence of the induction time as a function of the aliquot size.

Figure 4. Time course of the luminol luminescence in the luminol/ABAP system following the addition of red wine (I) aliquots. The wine was diluted 10^4 times prior to the aliquot addition: (A) 5 μL ; (B) 10 μL ; (C) 20 μL ; (D) 100 μL . The total volume of the sample was 1 mL. Curves (A) to (C) were obtained employing 2 mM ABAP. Curve (D) was obtained employing 10 mM ABAP, in order to attempt a better definition of the induction time. The initial reading was adjusted to a value of 100 under all the conditions employed.

of wines, both the TRAP (evaluated by the ABTS method) and the TAR values are nearly six times larger in red wines than in white wines.

A peculiar feature of Table 1 is the low distribution of the data. In fact, the results employing different yerba mate

Figure 5. Values of I_0/I derived from Curves (A) to (C) of Fig. 4 plotted against the size of the red wine aliquot.

infusions, when normalized by the absorbance of the sample at 323 nm, present a distribution of less than $\pm 9\%$. Also the fraction of fast decay, f_{fast} , is very similar for all the samples analyzed. This fraction, which in principle can be related to the presence of compounds with high reactivity towards the ABTS derived radical cations^{9,13}, is also very similar in red and white wines.

The results given in Table 2 indicate that with regard to yerba mate extracts, the TAR values are an average of 1.4

Table 2. TAR and TRAP values, in mM Trolox equivalents, obtained from the quenching of luminol chemiluminescence.

Beverage	Type	TAR	TAR/A ₃₂₃	TRAP	TRAP/A ₃₂₃
Yerba mate	tea bag (I)	4.7	10.0	3.1	6.6
	leaves (I)	4.5	13.6	3.1	9.4
	tea bag (II)	3.8	9.0	2.5	5.9
	leaves (II)	3.5	13.0	3.1	11.5
	tea bag (III)	5.6	10.6	3.4	6.4
	tea bag (IV)	6.1	12.3	3.8	7.6
	leaves (V)	2.3	12.1	2.2	11.6
	leaves (VI)	7.2	19.7	6.1	16.6
		4.7 ± 1.5	12.5 ± 3.1	3.4 ± 1.1	9.4 ± 3.4
Red wine	Sauvignon (VII)	37			
	Sauvignon (VIII)	33			
White wine	Sauvignon (VIII)	5.5			

times higher than the TRAP values. These results contrast with those obtained regarding the antioxidant capacity of blood plasma, where it was found that the TRAP values were considerably larger than the TAR values¹².

The differences between TRAP and TAR values obtained by the luminol technique, and between TRAP values obtained by both procedures can be accounted for in terms of the factors affecting both parameters. The TRAP value is defined by

$$\text{TRAP} = \frac{\sum n_i [X_i]}{n_{\text{Trolox}}} \quad (4)$$

where the equation is carried out for all the antioxidants present in the tested fluid, where n is the number of free radicals trapped by the antioxidant molecule, and $[X]_i$ is the concentration of the i -th antioxidant in the tested fluid. The value of the TRAP for a given fluid is then determined by the concentration of the antioxidants and their (n_i/n_{Trolox}) values. While for each phenol group (n_i/n_{Trolox}) can be considered nearly equal to one in the luminol based method, values higher than one have been obtained by a series of monophenols with regard to their capacity to bleach ABTS-derived radical cations. In fact, for a series of mono- and poly-substituted monophenols^{9,26}, it has been found that $n_i/n_{\text{Trolox}} = 1.52 \pm 0.34$, a value which could explain the larger TRAP values obtained by the ABTS procedure.

The TAR index is defined by

$$\text{TAR} = \frac{\sum k_i [X_i]}{k_{\text{Trolox}}} \quad (5)$$

where k_i is the reactivity of the i -th compound towards the radicals being trapped. In the present system, the radicals that can be trapped are the ABAB-derived and luminol-derived radicals. For the evaluation of the TRAP index, the

nature of the radical trapped is almost irrelevant. On the other hand, TAR values can be affected if the relative reactivity of the antioxidants and Trolox varies with the trapped radical. In the present system, we have shown that the radicals most likely to be trapped are the luminol derived radicals²⁷, and hence TAR indexes reflect the capacity of the additive to be engaged in processes of electron transfer to those radicals. This implies that extrapolation of TAR indexes to other situations must be carried out with caution.

The fact that in yerba mate the TAR values are higher than the TRAP values implies that some of the compounds in the extract must be considerably more reactive than Trolox (*i.e.*, $k_i > k_{\text{Trolox}}$). This result is the opposite of that found for blood plasma, where $\text{TAR} \ll \text{TRAP}$ is due to the predominance of uric acid, an antioxidant of relatively low reactivity²⁵. It is interesting to note that in red wine the TAR evaluated from the luminol data is larger than the TRAP evaluated from the ABTS data, indicating that in red wine as well compounds with higher reactivity than Trolox must be present.

In conclusion, the data reported in the present work indicate that both methods give similar and, in some cases, complementary results. In particular, the large antioxidant capacity of red wine and yerba mate is due to the presence of both very reactive and low reactive antioxidants, the latter in rather high concentrations. This is an ideal situation with regard to protection, since they can modulate the steady state free radical concentration (as indicated by the TAR) for fairly long periods (as indicated by the TRAP and the slow recovery of the luminescence).

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